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Atypical distyly in *Psychotria goyazensis* Mull. Arg. (Rubiaceae), an intramorph self-compatible species

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ABSTRACT

Distyly is a genetically controlled floral dimorphism, characterized by the reciprocal positioning of pin and thrum morphs, a heteromorphic incompatibility system and a balanced morph ratio (isoplethy). This study aimed to investigate the phenology, features of distyly, reproductive biology, and floral visitors of *Psychotria goyazensis* Mull. Arg. (Rubiaceae), an intramorph self-compatible species, in a tropical semideciduous forest in the municipality of Catalão, located in the state of Goiás, Brazil. The phenology was recorded for a sample of individuals, the floral morphology being described using fresh and fixed material. The incompatibility system was tested using controlled hand pollinations. Floral visitors were collected and identified. Blooming began in November 2009 and ended in April 2010. Fruits developed from December 2009 to March 2010. The population presented atypical distyly, because only plants with pin flowers were found. The intramorph and open pollinations set more fruits than did spontaneous self-pollinations. The main flower visitors were *Systropus* sp. (Diptera), as well as Hymenoptera and Lepidoptera species. Morphological variations in distyly can occur by breakdown/permutation of the gene that controls the polymorphism. However, some authors argue that, in Rubiaceae, genetic control of the incompatibility system can operate independently of the morphological factor, and species might respond in different ways to the same selective pressures.

Key words: heterostyly, monomorphism, *cerrado*, reproductive biology

Introduction

Heterostyly is genetically controlled floral polymorphism, characterized by reciprocal positioning of stigmas and anthers between morphs (reciprocal herkogamy), as well as by an intramorph self-incompatibility system (Ganders 1979). The most common manifestation of heterostyly is distyly, in which the two floral morphs, pin flowers (stigma above the anthers) and thrum flowers (stigma below the anthers), occur separately (Hamilton 1990). These morphs are usually found in similar proportions within populations (isoplethy); these features are controlled by genetic and ecological mechanisms, such as the incompatibility system and intermorph pollen transfer (Ganders 1979).

In some botanical families, the incompatibility system and floral morphs are controlled by a single genetic component, usually referred to as a supergene, whereby the thrum morph presents the heterozygous genotype and the pin morph presents the recessive homozygous genotype (Barrett 1990). However, some authors argue that, in certain families (especially Rubiaceae), the morphological and physiological integrity of the supergene differs from that seen in other families (Richards 1986; Lewis & Jones 1992; Barrett & Shore 2008; Consolaro *et al*. 2011). Some Rubiaceae genera, such as *Psychotria*, *Palicourea*, *Coccocypselum*, *Declieuxia*, *Sabicea*, *Guettarda* and *Manettia*, are known to be typically distylous but present anomalies or breakdown of distyly (Sobrevila *et al*. 1983; Hamilton 1990; Richards & Koptur 1993; Passos & Sazima 1995; Faivre & McDade 2001, Coelho & Barbosa 2003; Consolaro *et al*. 2009).

It is estimated that in Brazilian savanna (*cerrado*), distylous species represent approximately 41% of the Rubiaceae *taxa* (H. Consolaro, personal observation). In the Psychotrieae tribe, distyly seems to be a primitive feature and is taxonomically widespread, other reproductive systems in the tribe therefore being attributed to some evolutionary variation of distyly (Hamilton 1990). Because of different evolutionary and ecological implications, disturbance in the plant-pollinator relationship and genetic changes in the distyly, the floral structures and the frequency of the

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morphs can vary, resulting in the loss of one of the morphs (monomorphism) or a rearrangement of the reproductive structures, which can be placed at the same height (homostyly, Ganders 1979).

Plants show many different phenological strategies to avoid overlap between blooming and fruiting, as well as to compete for pollinators and seed dispersers, given that these adaptations can improve the fitness and survival of these species (Monasterio & Sarmiento, 1976; Bullock 1985). In recent decades, the number of studies on heterostyly and phenology of heterostylous species in the *cerrado* has been increasing (Monteiro *et al.* 1991; Coelho & Barbosa 2003; Coelho & Barbosa 2004; Consolaro *et al.* 2005; Toledo *et al.* 2007; Lenza *et al.* 2008; Oliveira 2008; Consolaro *et al.* 2009; Machado *et al.* 2010; Consolaro *et al.* 2011) and could be compared to those conducted in the Atlantic Forest (Passos & Sazima 1995; Lopes & Buzato 2005; Castro & Oliveira 2002; Castro *et al.* 2004; Teixeira & Machado 2004a, 2004b; Rossi *et al.* 2005; Pereira *et al.* 2006; Mendonça & Anjos 2006; Virillo *et al.* 2007, Fonseca *et al.* 2008). To increase understanding of the heterostyly system in the *cerrado*, this study aimed to investigate the phenology, distylous features, reproductive biology and floral visitors of *Psychotria goyazensis* Mull. Arg. (Rubiaceae).

Material and methods

Study site

The study was carried out from April 2009 to April 2010 in a forest fragment of approximately 26 ha in the municipality of Catalão, located in the state of Goiás, Brazil (18°9'36.595"S; 47°55'33,989"W). According to the Köppen classification system (Köppen 1958), the climate of the study area is type Aw, with a dry season from May to September and a rainy season from October to April.

Phenology

We marked 45 plants, with at least 5 m between individuals. During the reproductive phase (flowering and fruiting), observations were made on a weekly basis, whereas they were made once every two weeks during the vegetative phase. The development of flower buds, the appearance of open flowers (blossoming) and fruiting were recorded. To evaluate the phenological phases, we employed the activity and intensity indices devised by Bencke & Morellato (2002). The activity index analyzes the presence or absence of each phenological phase (flower buds, flowers and fruits), evaluating the synchrony of the phenological events among the plants. The intensity index uses the classes established by Fournier (1974), showing the peak intensity of each phenological event on a five-point scale: 0 = 0%; 1 = 25%; 2 = 50%; 3 = 75%; and 4 = 100%. Phenological patterns were classified using the categories described by Newstron *et al.* (1994).

Floral biology

Forty pre-anthesis buds were marked on 10 random plants (4 buds per plant). The floral longevity, the release of pollen by the anthers and the beginning of anthesis were recorded. Anthesis onset was defined as the beginning of the opening of corolla lobes, and the end of floral longevity was defined as a loss of brightness and a change in the color of the corolla. The release of pollen was determined by observation of loose pollen on the anthers. Stigma receptivity was determined by the separation of the stigma lobes (n = 16), which is usually suitable for studies of Rubiaceae (Coelho & Barbosa 2003, Consolaro *et al*. 2005, Consolaro *et al.* 2009, Consolaro *et al.* 2011).

Distyly and incompatibility system

Five flowers per individual were collected from 20 plants. The lengths of the corolla tube, stamens, pistil and stigma lobes were measured under a stereomicroscope and are expressed as mean ± standard error of mean. The fruit set resulting from controlled hand pollination was used in order to determine the incompatibility system. In 27 plants, flower buds in pre-anthesis (n = 172) were isolated and bagged. When the flower buds opened, the following treatments were applied: self-pollination (exposure to pollen from the same flower, n = 41); intramorph cross-pollination (exposure to pollen from flowers of different plants of the same morph, n = 42); spontaneous self-pollination (flowers tagged and bagged, n = 48); and control (flowers marked and exposed to open pollination, n = 41). We did not expose flowers to intermorph cross-pollination, because the studied population was strictly monomorphic. The index of self-incompatibility (ISI) was calculated as the ratio between the proportion of fruit production by selfing and that resulting from intramorph cross-pollinations. An ISI greater than 0.25 was considered indicative of self-compatibility (adapted from Bullock 1985).

Floral visitors

During four days at the blooming season, we observed the flower visitors of 14 plants. Observations were intercalated at the morning and at the afternoon (from 06:00h to 16:00h), with four hours of duration and a total of 24h of observation. The floral visitors were categorized as nectar robbers (those "robbing" nectar from outside the corolla, i.e., without making contact with the anthers or stigma) or pollinators (those taking nectar or pollen and making contact with the anthers and stigma). The frequency and type of approach to the flowers were also recorded, and the visitors were collected and identified. The reproductive efficiency index was calculated as the ratio between the control (open pollination) fruit set and the intramorph cross-pollination fruit set (adapted from Ruiz & Arroyo 1978).

Statistical analysis

A two-tailed *t-test* was used in order to identify differences between the anthers and stigma heights, and a chi-square test was applied in order to compare the fruit-set frequency between the hand pollinations treatments. Data analysis was performed using BioEstat 3.0 software (Ayres *et al.* 2003).

Results

Phenology

The reproductive phenology of *P. goyazensis* showed an annual intermediate pattern (*sensu* Newstron *et al.* 1994): it presented only one flowering cycle over a year with an approximate duration of four months (Fig. 1). Buds began to appear in November and continued to appear until the end of February, and flowering began at the end of November 2009 and continued until April 2010, both phenological phases occurring during the rainy season. During the flowering peak, we noted that the proportion of plants with open flowers in the population (25.58%) was substantially lower than the observed proportion of plants showing floral buds (73.78%). As can be seen in Fig. 1, fruit production occurred concurrently with flowering, beginning in December 2009, the fruits maturing in March 2010 (at the beginning of the dry season). At all stages observed, the synchrony between individuals was approximately 41.33%, although the proportion of plants with evidence of phenological phases ranged from 11.71% to 100%.

Floral biology

Psychotria goyazensis has white tetramerous or pentamerous flowers (Fig. 2), arranged in cymose inflorescences. They lasted approximately 12 h, presenting nectar and pollen as floral resources. Inside the corolla, there is a ring of trichomes arranged in the middle portion of the tube, above which are the four stamens. Anthesis began at 05:00h flowers typically being fully opened by 06:00h, at which time stigmas became receptive and pollen release began.

Distyly and isoplethy

Psychotria goyazensis presented a case of atypical distyly, because only the pin morph was found in the population (pin monomorphism). The pin morph flowers had a mean corolla tube length of approximately 3.1 ± 0.33 mm, mean stamen height of 2.77 ± 0.02 mm, mean pistil height of 3.27 ± 0.03 mm and mean stigma lobe length of 1.9 ± 0.32 mm. The difference among the heights of the reproductive structures was significant ($t_{2,93}$=16.48, p<0.01), characterizing the pin morph. The mean anther-stigma separation was 0.5 ± 0.03 mm, with an apparent variation in herkogamy between flowers. A few flowers presented pistils at approximately the same level as or even slightly below the anthers (Fig. 3).

Incompatibility system

Psychotria goyazensis showed intramorph and self-compatibility, with an index of self-incompatibility of 0.96. As can be seen in Tab. 1, there was no significant difference between self-pollination and intramorph cross-pollination in terms of fruit set (χ^2=0.017, p>0.05). We also found that the fruit set resulting from spontaneous self-pollination did not differ significantly from that resulting from the cross-pollination and open pollination (control) treatments (reproductive efficiency index=1.19, χ^2=0.38, p=0.61). Fruit set was observed 15 to 20 days after controlled hand pollinations.

Floral visitors

Only insects were observed visiting the flowers of *P. goyazensis*. Most of the visits (52%, n = 60) were made by Diptera (*Systropus* sp., mimics of Hymenoptera, 43% of the visits being made by flies of the Conopidae and Syrphidae families), bees (Halictidae, 2 species), wasps (Polistinae, 2 species), and butterflies (Nymphalidae and Danainae). The visits were legitimately performed, meaning that the visitors apparently touched the anthers and stigmas with the front of their body and were primarily seeking nectar. We identified no nectar robbers. Although visits were recorded throughout the day, they were more frequent at times when there was a higher incidence of sunlight on plants (between 9:00h and 11:00h). Pollinators visited many flowers on the same plant and flowers on plants that were closer to each other.

Discussion

The blooming pattern of Rubiaceae and *Psychotria* species in the *cerrado* often seems to be linked to the rainy season in this ecosystem (Martin-Gajardo & Morellato 2003, Lopez & Buzato 2005, Consolaro *et al.* 2011). However, the bloom period and the fructification of many species can result not only from abiotic selective pressures, such as the climate system, but also as a way to enhance survival strategies when the species will be able to disperse and germinate its seeds (Sarmiento & Monasterio 1983). The synchrony among the studied plants followed the intensity of occurrence of the phenological phases observed, a pattern found in other *Psychotria* species (Augspurger 1983; Coelho & Barbosa 2004). The timing of a phenological phase, such as flowering and fruiting, is closely related to endogenous factors and abiotic factors that influence the physiology of plants (Bencke & Morellato 2002). Nevertheless, in an ecological context, this fact is important because it contributes to the reproductive process of the species, increasing the

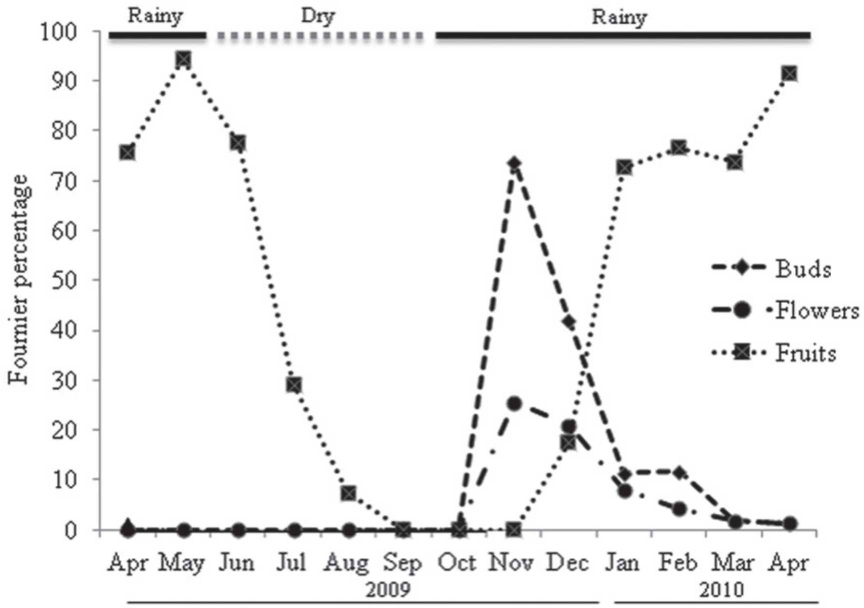

Figure 1. Phenology as proportional intensity for each phenophase (budding, flowering and fruit production) of *Psychotria goyazensis* Mull. Arg. in a forest fragment in the municipality of Catalão, state of Goiás, Brazil.

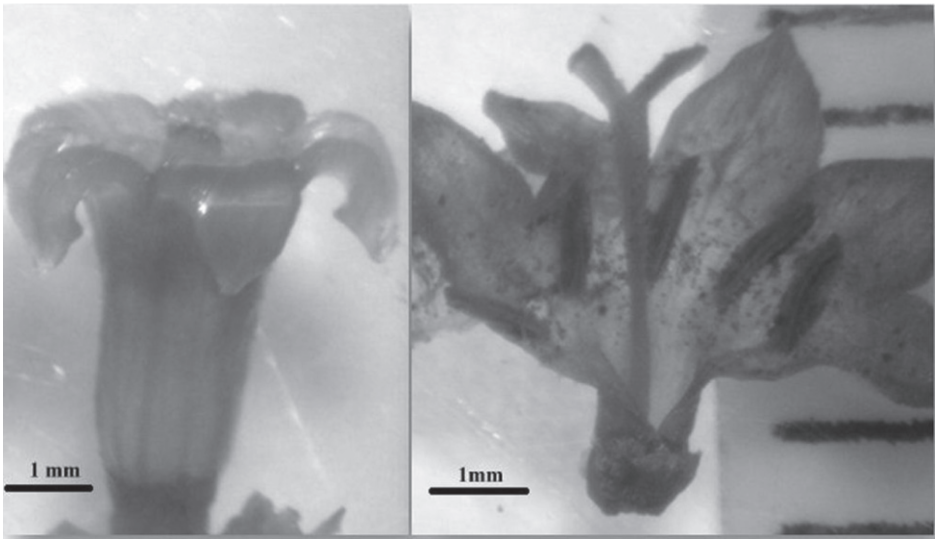

Figure 2. *Psychotria goyazensis* Mull. Arg. flowers. A: Corolla of pin flower. B: Reproductive structures of pin flower in a forest fragment in the municipality of Catalão, state of Goiás, Brazil.

power of attraction and the maintenance of pollinators and seed dispersers in the habitat.

In the study area, we recorded five other *Psychotria* species that bloom simultaneously with *P. goyazensis*, to which they are also similar in terms of morphology and floral biology: *P. deflexa*, *P. prunifolia*, *P. hoffmannseggiana*, *P. trichophoroides* and *P. capitata* (H. Consolaro, personal observation). The coexistence of plants whose flowering times overlap could have a facilitating effect on pollination, increasing the attraction of pollinators to neighboring species (Feldman *et al.* 2004). However, it can also expose these species to interspecific competition for pollinators, having a negative impact on pollen transfer and fruit production (Pleasants 1980).

Because the number of studies on the reproductive biology of *Psychotria* species is increasing, it is often possible to find the occurrence of monomorphic populations, especially pin species (Ganders 1979, Sakai & Wright 2008; Consolaro *et al.* 2011). The same pin monomorphism found in *P. goyazensis* has been also reported in *P. carthagenensis* (Con-

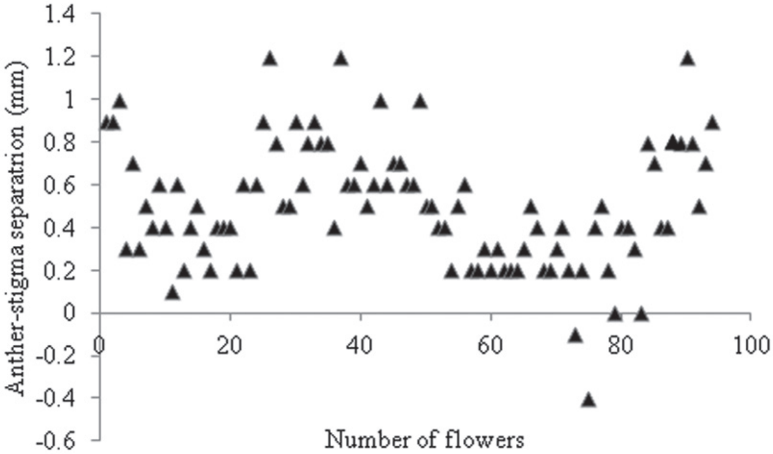

Figure 3. Anther-stigma separation of *Psychotria goyazensis* Mull. Arg. flowers in a forest fragment in the municipality of Catalão, state of Goiás, Brazil.

solaro *et al*. 2011) and *P. graciliflora*, *P. hoffmannseggiana*, *P. micrantha* and *P. tenuifolia* (Sakai Wright & 2008). Four of the five *Psychotria* species in the study area are typically distylous (*P. hoffmannseggiana*, *P. capitata*, *P. deflexa* and *P. trichophoroides*), although *P. prunifolia* is also pin monomorphic (E. Rodrigues, personal observation). A recent phylogenetic analysis showed that such unbalanced morph ratios can arise independently (Sakai & Wright 2008). Such ratios can also be observed among populations of the same species (Koch *et al*. 2010; Consolaro *et al*. 2011; Farias *et al*. 2012), as well as in congeneric species (Consolaro *et al*. 2009), although other species are relatively stable (e.g. Machado et al. 2010). In *P. goyazensis*, we were unable to determine how widespread pin monomorphism is among its populations.

There is no concrete information about how the genetic control of distyly in Rubiaceae works, but studies of other families indicate that a diallelic system organized as a supergene controls the morphological and incompatibility features of distyly (Richards 1986; Lewis & Jones 1992; Barrett & Shore 2008). When a breakdown of distyly occurs by a recombination in the supergene (Lewis & Jones 1992), the most common result would be the formation of homostylous self-compatible flowers, with reproductive structures at the same height (Ganders 1979; Consolaro *et al*. 2009). The breakdown of distyly can also be correlated with the ploidy level in some Rubiaceae species (Naiki &Nagamasu 2004; Naiki 2012). However, it is known that a gain in compatibility can occur without rearrangement of the reproductive structures (Sobrevila *et al*. 1983; Barrett 1988; Consolaro *et al*. 2011), which indicates that the action of the supergene in Rubiaceae is not very clear. The occurrence of pin monomorphism and self-compatibility in the studied population of *P. goyazensis*, as shown in *P. carthagenensis* (Consolaro *et al*. 2011), supports the idea that in Rubiaceae the control of the incompatibility system could act independently of the morphological factor.

The development of an autogamous reproductive mechanism becomes advantageous when pollination service and compatible pollen transfer is lost and seed production is deficient (Schoen *et al*. 1996). Distylous species rely on an accurate intermorph pollen transfer due to the incompatibility system, so that the loss or laxity of this system becomes advantageous in case of disturbance of the plant-pollination relationship (Ganders 1979).

In the present study, *P. goyazensis* flowers were mainly visited by flies, a rare situation for this genus that has bees as its main pollinators (Bawa & Beach 1983; Castro & Oliveira 2002). Variations in the type of pollinator among congeneric species might have a negative impact on intermorph pollen transfer, because the floral architecture might not favor contact with flower visitors (Consolaro *et al*. 2009), especially Syrphidae, which have a smaller body size and distinct behavior (Proctor *et al*. 1996). In addition, even when Diptera species visit the flowers, the visits happen during specific periods of the day (between 08:00h and 12:00h), many Syrphidae species having been shown to present such behavior (Weems 1953). With visits of pollinators to *P. goyazensis* flowers restricted to specific times of day, the pollination service could have become limited and insufficient. The irregular frequency of visits and instability in the pollen transfer might have favored the loss of incompatibility in the studied population.

Because there was a comparatively normal fruit set after spontaneous self-pollination treatment, we cannot exclude the possibility of apomixis in this population. However, in tiny flowers, such as those of *P. goyazensis*, the anther-stigma separation might be insufficient to avoid spontaneous self--pollination. Their size also renders emasculation and agamospermy treatments impractical. Even herkogamy, which results in significant differences in height of the reproductive structures, might not be sufficient to prevent deposition of self-pollen on the pistil. Dai & Galloway (2011) suggested that there are cases in which herkogamy does not function

Table 1. Fruit set of controlled hand pollination experiments of *Psychotria goyazensis* Mull. Arg. (Rubiaceae) flowers from a forest fragment in the municipality of Catalão, state of Goiás, Brazil.

Treatment (n of flower buds)	Fruiting n (%)*
Self-pollination (n = 41)	29 (70.73)^{a}
Intramorph cross-pollination (n = 42)	30 (71.42)^{a}
Spontaneous self-pollination (n = 48)	19 (39.58)^{b}
Open-pollination control (n = 41)	35 (85.36)^{a}

*Different letters indicate a significant difference between values ($p<0.05$).

as a system that prevents self-pollination but only a way to increase pollen transfer and outcrossing, which could be the case among flowers of *P. goyazensis*.

The overlap between *P. goyazensis* and the five other *Psychotria* species, in terms of blooming time; together with the fact that *P. goyazensis* has small Diptera as its main pollinators and a limited time for pollination as a function of the foraging activity of the floral visitors, as well as having tiny flowers, might have contributed to the occurrence of variations in the reproductive system of the studied population. However, it was not possible to establish pin monomorphism or self-compatibility as features of the studied species, because the anomalies might be restricted to this population. Pin and thrum flowers of *P. goyazensis* have been described from herbarium material (Delprete 2010), so it is possible that isoplethic and truly distylous populations occur elsewhere. For Rubiaceae, especially in studies of *Psychotria*, the genetic control of the incompatibility system and the floral morphology by a supergene does not seem to be well-defined. Therefore, further specific studies in this family are needed in order to improve understanding of distyly and its variations in the Rubiaceae family, which presents a larger number of distylous species than does any other family of angiosperms.

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